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Heparinase I-specific disaccharide unit of heparin is a key structure but insufficient for exerting anti-prion activity in prion-infected cells

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ABSTRACT

Glycosaminoglycans reportedly play important roles in prion formation, but because of their structural complexity, the chemical structures affecting prion formation have not been fully evaluated. Here, we compared two types of low molecular weight heparins and found that heparinase I-sensitive structures influenced anti-prion activity in prion-infected cells. Surface plasmon resonance analyses showed significant binding of a representative heparinase I substrate disaccharide unit, GlcNS6S-IdoA2S, to recombinant prion protein (PrP) fragments, such as full-length PrP23–231 and N-terminal domain PrP23–89, but not to PrP89–230. This binding was competitively inhibited by heparin or pentosan polysulfate, but not by Cu^{2+}. These PrP binding profiles of the disaccharide unit are consistent with those previously reported for heparin. However, synthetic compounds comprising disaccharide unit alone or its multimers exhibited no anti-prion activity in prion-infected cells. Consequently, the findings suggest that the heparin disaccharide unit that binds to the N-terminal region of PrP is a key structure, but it is insufficient for exerting anti-prion activity.

1. Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders, including Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy and scrapie in animals. These diseases are characterized by deposition of an abnormal isoform of prion protein, which is the main component of prion, in the central nervous system and lymphoreticular system [1]. Prion protein (PrP) is converted from the normal cellular isoform to the abnormal isoform by still enigmatic mechanism. The abnormal PrP, which has protease resistance and detergent insolubility, is usually detected as protease-resistant PrP (PrPres) after treatment with proteinase K in immunoblotting analysis. The normal cellular PrP is a glycosylphosphatidylinositol-anchored cell surface membrane protein with two N-linked glycosylation sites.

Previous studies have suggested that glycosaminoglycans (GAGs), which exist endogenously on the cell surface and extracellular matrix, play important roles in prion formation and infection [2]. These molecules exhibit paradoxical effects *in vitro*. Some researchers have proposed a facilitating action on the conversion of normal to abnormal PrP [3–5], whereas others have suggested a protective action against the conversion [6,7]. Conversely, GAGs always show inhibitory activities against abnormal PrP formation in prion-infected cells [8], presumably by blocking PrP interaction with endogenous GAGs [9] or by decreasing the cell surface PrP content [10]. GAGs and polyanionic glycans also effectively prolong the incubation time of the disease in peripherally prion-infected animal models [11,12]. Particularly, pentosan polysulfate, a highly sulfated polysaccharide administered into the cerebral ventricles to bypass the blood–brain barrier [13] has been used in clinical trials for prion disease [14,15].

Previous studies of recombinant PrP fragments or synthetic PrP peptides have demonstrated that PrP interacts with GAGs through

Abbreviations: GAG, glycosaminoglycan; Glc, glucose; GlcNS6S-IdoA2S, (2-deoxy-2-sulfoamido-6-O-sulfo-α-D-glycopyranosyl)-(1–4)-O-(2-O-sulfo-α-L-idopyranosyluronic acid); IdoA, iduronic acid; LMWH, low molecular weight heparin; PrP, prion protein; PrPres, protease-resistant abnormal PrP; SPR, surface plasmon resonance.

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the N-terminal positive charge cluster [16] or through the three regions that include this positive charge cluster [17]. Warner et al. [17] reported that 2-O-sulfate groups but not 6-O-sulfate groups in a representative GAG, heparin, are essential for interaction with PrP. However, the chemical structures in GAGs necessary for anti-prion activity have not been fully evaluated. Detailed analyses of the anti-prion active components in GAGs are difficult because GAGs are heterogeneous in terms of composition, degree of modification, and molecular length.

Here, using low molecular weight heparins (LMWHs) prepared by two different methods, we show that heparinase I-sensitive chemical structures are important for anti-prion activity in prion-infected cells. We characterize the binding profile of a representative heparinase I substrate disaccharide unit to PrP. We also examine whether synthetic compounds composed of this disaccharide unit have sufficient anti-prion activity in prion-infected cells and discuss the significance of the disaccharide unit in anti-prion activity.

2. Materials and methods

2.1. Preparation of LMWHs and fractionation

LMWHs were prepared by depolymerization of porcine mucosal heparin (Nacalai Tesque Inc., Kyoto, Japan) and subsequent fractionation according to molecular weight using gel permeation chromatography (GPC; Sephadex G-50; Pharmacia, Uppsala, Sweden) as previously described [18]. Two methods for depolymerization were used: heparinase I digestion [19] and $NaIO_4$ treatment [20]. The molecular weight of LMWHs was estimated using heparin oligosaccharide standards of known molecular weight by GPC–high performance liquid chromatography (HPLC) on an analytical column (Asahipak GFA-3000; Asahi Chemical Ind. Co. Ltd., Osaka, Japan) in 200 mM phosphate buffer (pH 7.0) containing 0.3 M sodium chloride. Fractionated heparins were subjected to unsaturated disaccharide analysis according to the method reported by Yoshida et al. [21].

2.2. Anti-prion activity assay in cell culture

Mouse neuroblastoma cells that had been infected persistently with the RML prion strain (ScN2a cells) were used as a cellular model of prion infection [22]. The anti-prion activity of study compounds was measured by assaying the content of PrPres in ScN2a cells that were treated with the compounds for 3 days, using immunoblotting as previously described [23–25]. PrP was detected using a monoclonal antibody, SAF83, which recognizes residues 126–164 of PrP (1:5000; SPI-Bio, Massy, France). The toxicity of each compound was assessed based on the cell growth rate.

2.3. Preparation of sugar chain-immobilized chips (sugar chips)

GlcNS6S-IdoA2S-Glc was prepared and conjugated with a monovalent linker molecule to prepare a ligand-conjugate for the immobilization of GlcNS6S-IdoA2S on gold-coated chips, as previously described [26]. The sugar chips were stored under refrigeration until use.

2.4. Recombinant mouse PrP fragments

The production, purification, and characterization of mouse recombinant PrPs were performed as previously described [27,28], with some modifications. Briefly, PrP23–231 was precipitated from the solubilized inclusion body extract by dialysis against TN buffer (20 mM Tris [pH 7.5], 300 mM NaCl). The precipitant was resolubilized in 6 M urea-containing TN buffer, and then it was affinity purified using an Ni-NTA column (Qiagen Inc., Venlo, Netherlands) and dialyzed against 20 mM sodium acetate (pH 5.0). PrP23–89 was affinity purified from the solubilized inclusion body extract using the Ni-NTA column (Qiagen Inc.). Next, it was fractionated by RP–HPLC (10 × 250 mm, C_{18}; Nacalai Tesque Inc.) and lyophilized. For PrP89–230 purification, the solubilized inclusion body extract was dialyzed against water containing 0.1% formic acid. Next, PrP89–230 was fractionated by RP–HPLC (10 × 250 mm, C_4; Nacalai Tesque Inc.) and lyophilized.

2.5. Measurement of PrP binding to GlcNS6S-IdoA2S

Surface plasmon resonance (SPR) sensorgrams were recorded as previously described [29,30] using an SPR biosensor instrument (Nanosensor; Moritex Corp., Yokohama, Japan) equipped with a sugar chip as described above. Binding of PrP was detected as the difference in the resonance angle. For a competition study of PrP binding, PrP23–231 was preincubated with heparin or pentosan polysulfate before being loaded onto the sugar chip.

2.6. Compounds containing GlcNS6S-IdoA2S

Compounds containing the GlcNS6S-IdoA2S disaccharide unit alone or its multimers were synthesized at SUDx-Biotec Co. (Kagoshima, Japan) using the building block of GlcNS6S-IdoA2S disaccharide, as previously described [31,32].

3. Results

3.1. Anti-prion activity in prion-infected cells

LMWHs with molecular weights of 10,500, 7000, and 2400 (corresponding to 15, 10, and 4 disaccharide units, respectively) were prepared using two depolymerization methods and subsequent size fractionation. They were analyzed for anti-prion activity using ScN2a cells and were compared mutually. Depolymerization using heparinase I specifically disrupts the disaccharides GlcNS(±6S)-IdoA2S, especially GlcNS6S-IdoA2S [33,34]. GlcNS6S-IdoA2S is more susceptibly disrupted than GlcNS-IdoA2S because 6-O-sulfation of GlcNS6S enhances enzyme activity. However, depolymerization by $NaIO_4$ treatment, which selectively cleaves 1,2-diols, retains the disaccharide structure GlcNS6S-IdoA2S (Fig. 1A).

Although the disaccharide unit GlcNS6S-IdoA2S is not traceable by unsaturated disaccharide analysis, the difference between heparinase I- and $NaIO_4$-depolymerized LMWHs was reasonably demonstrated in the data for unsaturated disaccharide analysis (Table 1). Particularly, in smaller LMWHs (7000 and 2400), depolymerization with $NaIO_4$ reduced the content ratio of IdoA-sugar disaccharides (ΔDiHS-0S, -6S, and -diS_1 in Table 1), and depolymerization with heparinase I dramatically reduced the content ratio of tri-sulfonated disaccharides such as IdoA2S-GlcNS6S and GlcA2S-GlcNS6S (ΔDiHS-triS in Table 1).

The $NaIO_4$-depolymerized LMWHs inhibited the formation of abnormal PrP (PrPres) by 50% at 0.2–0.6 μg/mL (Fig. 1B). In contrast, heparinase I-depolymerized LMWHs with molecular weights of 7000 and 2400 did not inhibit the formation of abnormal PrP, even at much higher concentrations (Fig. 1C). Although heparinase I-depolymerized LMWH with a molecular weight of 10,500 showed similar anti-prion activity to the $NaIO_4$-depolymerized LMWH of the same molecular weight (Fig. 1D), heparinase I digestion of the $NaIO_4$-depolymerized LMWH resulted in an obvious reduction in anti-prion activity to a level similar to that observed on heparinase I digestion of the original non-depolymerized heparin sample

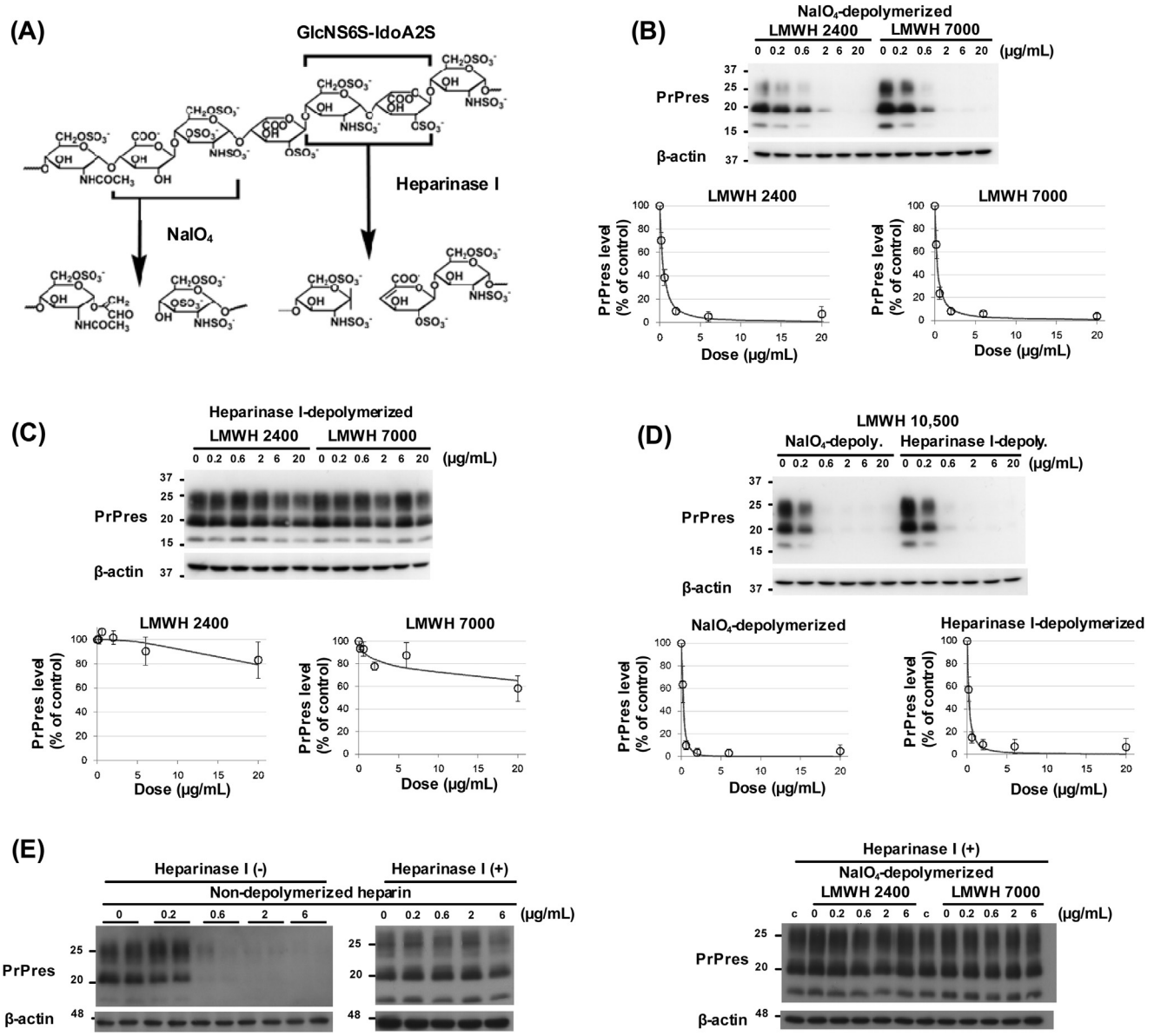

Fig. 1. Comparison of anti-prion activities of low molecular weight heparins (LMWHs) prepared using two depolymerization methods and subsequent molecular weight fractionation. (A) Chemical structures of heparin, the GlcNS6S-IdoA2S disaccharide unit, and depolymerized products. Representative substrate for depolymerization with $NaIO_4$ or heparinase I is shown. (B–E) Immunoblot analyses of protease-resistant abnormal prion protein (PrPres) in prion-infected ScN2a cells treated with LMWHs. ScN2a cells were incubated at concentrations shown at the top of the blots. Graphic data are averages and standard deviations for triplicate immunoblot signals. (B) ScN2a cells treated with $NaIO_4$-depolymerized LMWHs, (C) ScN2a cells treated with heparinase I-depolymerized LMWHs, (D) ScN2a cells treated with a larger-size $NaIO_4$-depolymerized LMWH or heparinase I-depolymerized LMWH, (E) ScN2a cells treated with either non-depolymerized heparin or $NaIO_4$-depolymerized LMWHs that had been digested or non-digested with heparinase I. Molecular size markers in the left side of the immunoblots are shown in kilodaltons. β-actin signals are shown as controls for the integrity of samples used for PrPres assay. The "c" denotes the control treated with heparinase I digestion buffer.

(Fig. 1E). These results suggest that heparinase I-sensitive chemical structures play a central role in anti-prion activity.

3.2. Binding of PrP23–231 to GlcNS6S-IdoA2S

In further studies, we focused on GlcNS6S-IdoA2S rather than GlcNS-IdoA2S for three reasons: GlcNS6S-IdoA2S is more susceptibly disrupted by heparinase I than GlcNS-IdoA2S [33,34]; prion formation is greatly influenced by sulfation density in GAGs [9,35]; and GlcNS-IdoA2S is possibly cleaved by $NaIO_4$ treatment. The interaction between recombinant PrP and GlcNS6S-IdoA2S was examined by SPR using a sugar chip in which the disaccharide unit was anchored via a spacer glucose-derived unit and a monovalent linker unit (Fig. 2A, inset). Next, 10 μM of full length PrP23–231 solution was loaded onto the sugar chip. A typical binding saturation curve was observed (Fig. 2A). As a control experiment, a sensor chip bearing only the linker generated no significant signal for binding with PrP23–231 (data not shown), indicating that the non-specific binding of PrP23–231 to the sensor chip was below the detection limit of this system.

After reaction of PrP23–231 solution with the sugar chip, the unbound material was washed out thoroughly and tryptic digestion of bound material on the sugar chip was performed. This treatment reduced the sensorgram intensity and released digested peptide fragments from the chip, corresponding to PrP25–37, 38–48, 148–155, 194–203, 220–228, and 220–229, as

Table 1
Unsaturated disaccharide analysis data of LMWHs.

Molecular weight	Heparinase I-depolymerized LMWHs			$NaIO_4$-depolymerized LMWHs		
	10,500	7000	2400	10,500	7000	2400
ΔDiHS-0S (%)	3.3	22	17	0	0	0
ΔDiHS-6S (%)	21	23	20	0	0	0
ΔDiHS-NS (%)	1.6	0	0	7.3	3.4	2.8
ΔDiHS-diS_1 (%)	5.3	18	21	11	0	0.2
ΔDiHS-diS_2 (%)	7.1	10	11	11	7.4	6.6
ΔDiHS-triS (%)	62	27	32	71	89	90

ΔDiHS-0S, 2-acetamido-2-deoxy-4-O-(4-deoxy-α-L-threo-hex-enopyranosyluronic acid)-D-glucose.
ΔDiHS-6S, 2-acetamido-2-deoxy-4-O-(4-deoxy-α-L-threo-hex-enopyranosyluronic acid)-6-O-sulfo-D-glucose.
ΔDiHS-NS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-glucose.
ΔDiHS-diS_1, 2-acetamido-2-deoxy-2-sulfamino-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose.
ΔDiHS-diS_2, 2-deoxy-2-sulfamino-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-D-glucose.
ΔDiHS-triS, 2-deoxy-2-sulfamino-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose.

demonstrated by mass spectrometry (data not shown). This result indicated that the sensorgram signal was caused specifically by PrP23–231.

The intensities of the sensorgrams increased in accordance with the loading concentrations of PrP23–231. Sensorgrams were analyzed assuming a first-order equilibrium reaction between PrP and GlcNS6S-IdoA2S. An apparent dissociation constant K_D was calculated as 9.7 ± 3.0 μM in which the kinetic constants were 0.0018 ± 0.00025 s^{-1} μM^{-1} PrP and 0.018 ± 0.0032 s^{-1} μM^{-1} PrP for dissociation (k_d) and association (k_a), respectively. Under higher salt concentrations in the binding buffer, the intensities of sensorgrams of PrP23–231 decreased (Fig. 2B). At a concentration of 400 mM of NaCl, the sensorgram intensity decreased to 50% of that at 140 mM.

To investigate the specificity of the interaction between PrP23–231 and the disaccharide unit, PrP23–231 was pre-incubated with heparin or pentosan polysulfate before loading onto the sugar chip. The sensorgram intensities decreased in accordance with doses of heparin or pentosan polysulfate (Fig. 2C). Either compound at 0.80 μg/mL decreased the intensity of the sensorgram to 50% of that without a compound.

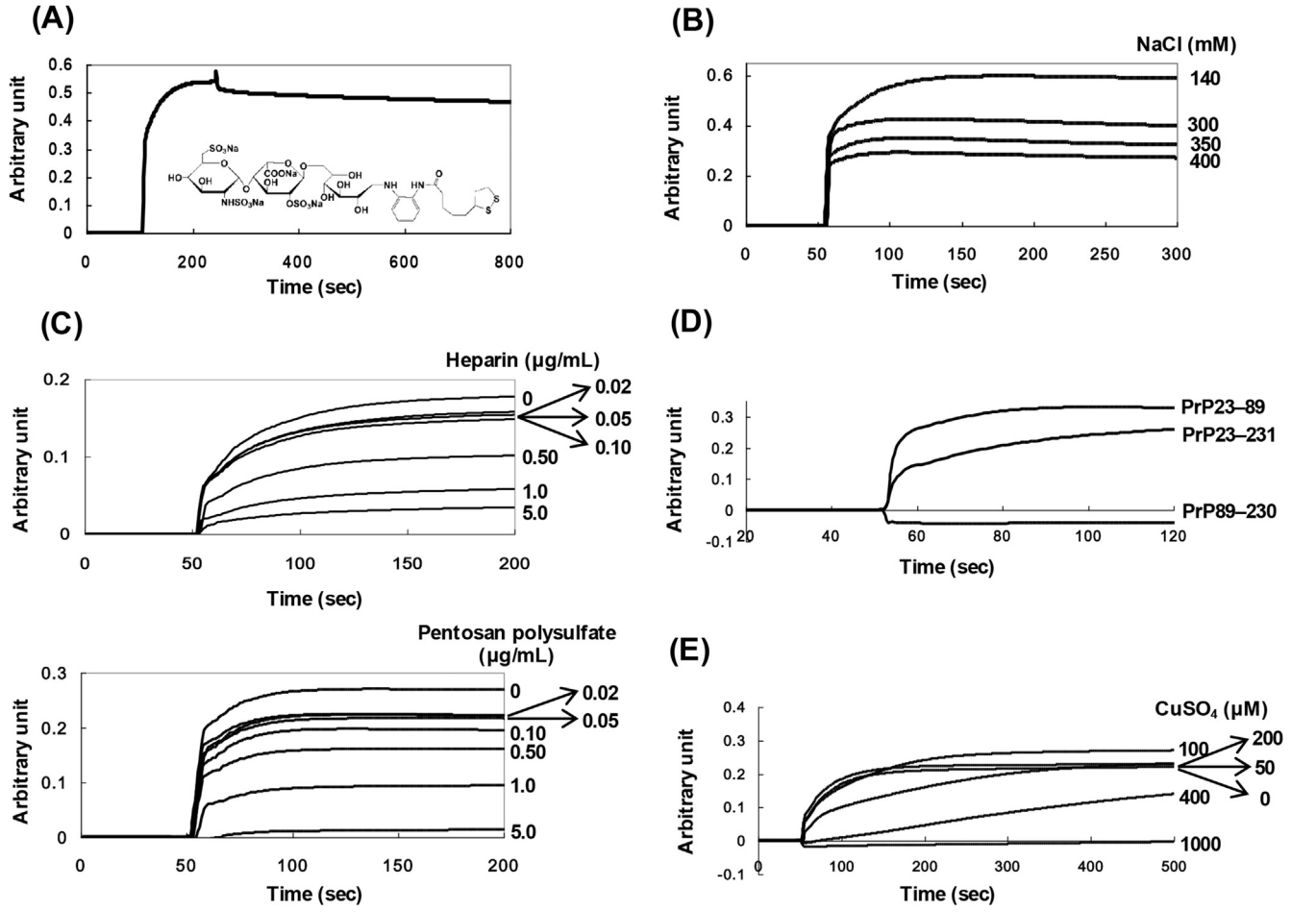

Fig. 2. Binding profiles of recombinant prion protein (PrP) fragments to the sugar chip bearing GlcNS6S-IdoA2S. (A) Full length PrP23–231 (10 μM) was analyzed on the sugar chip bearing GlcNS6S-IdoA2S. After the binding reaction for 150 s, unbound PrP23–231 was washed out. The inset shows the chemical structure of the sugar chain anchored to the sensor chip. (B) Full length PrP23–231 (10 μM) was loaded under the designated concentrations of NaCl. (C) Full length PrP23–231 (10 μM) was preincubated with the designated concentrations of heparin or pentosan polysulfate before being loaded onto the sugar chip. (D) Full length PrP23–231 and domain-truncated PrP fragments (PrP23–89 and PrP89–230) were analyzed on the sugar chip. Concentrations of PrP fragments were 10 μM for PrP23–231, 10 μM for PrP23–89, and 11 μM for PrP89–230. (E) Full length PrP23–231 (10 μM) was loaded under the designated concentrations of $CuSO_4$.

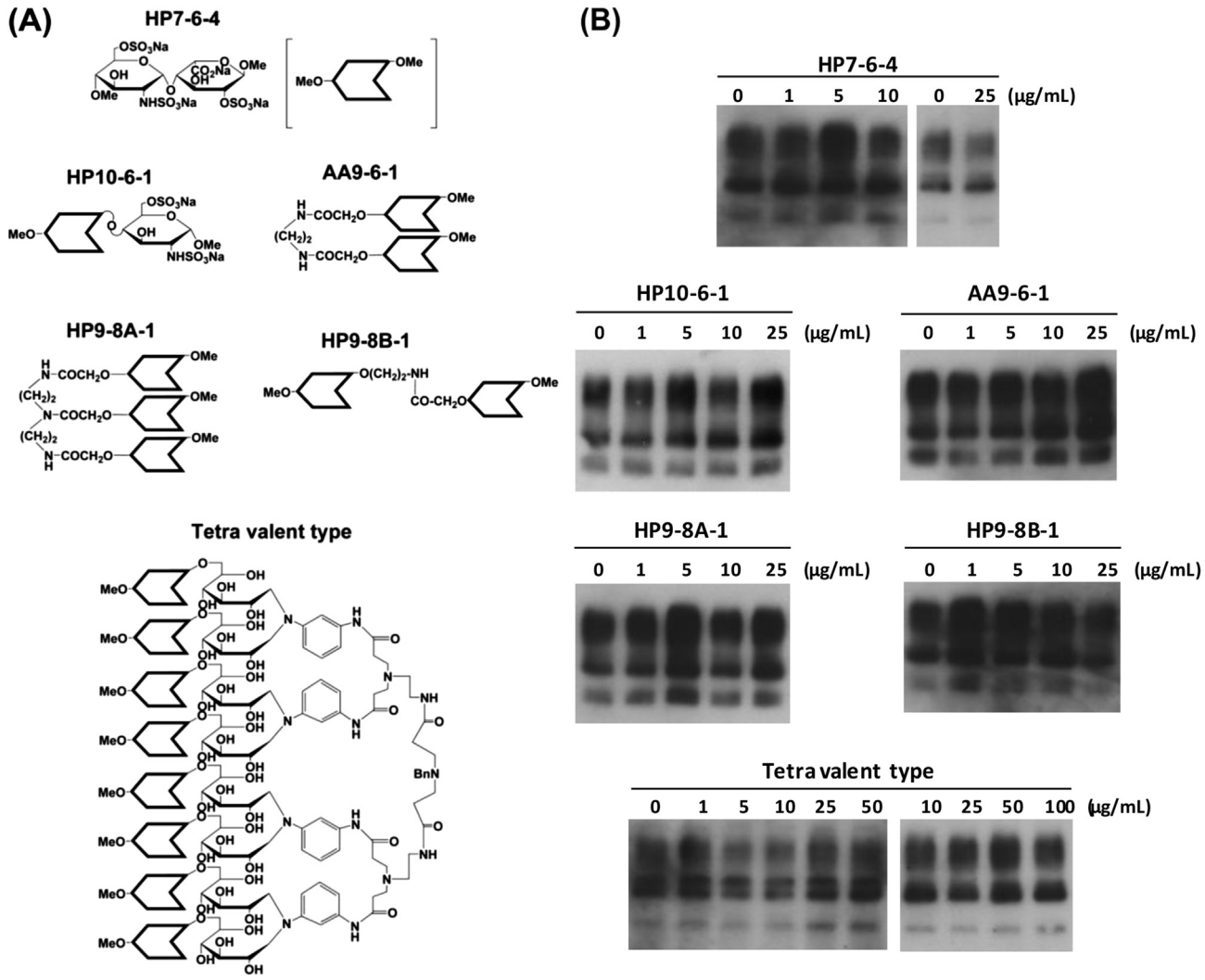

Fig. 3. Anti-prion activities of synthetic GlcNS6S-IdoA2S-related compounds. (A) Chemical structure of synthetic GlcNS6S-IdoA2S-related compounds. (B) Representative immunoblot data of PrPres in ScN2a cells treated with synthetic compounds. Cell toxicity was not observed at any indicated dosage tested here.

3.3. PrP binding domain to GlcNS6S-IdoA2S

To determine the binding site of PrP23–231 to the disaccharide unit, domain-truncated PrP fragments, PrP23–89 and PrP89–230, were prepared and analyzed. PrP23–231, PrP23–89, and PrP89–230 were loaded onto sugar chips at concentrations of 10, 10, and 11 μM, respectively. Positive binding saturation curves were observed for PrP23–231 and PrP23–89 (Fig. 2D). However, no significant signal for PrP89–230 binding to the disaccharide unit was observed. These results indicate that the binding site of the disaccharide unit is included in the PrP23–89 domain. The intensities of sensorgrams increased in accordance with loading concentrations of PrP23–89 (data not shown). Analysis of the sensorgrams revealed values for K_D, k_d, and k_a of 16 ± 3.0 μM, 0.060 ± 0.0054 s^{-1} μM^{-1} PrP, and 0.0038 ± 0.00040 s^{-1} μM^{-1} PrP, respectively.

Because PrP23–89 contains an octapeptide repeat sequence that can bind to Cu^{2+} [36], we examined whether Cu^{2+} influences the interaction between PrP and the disaccharide unit. Addition of Cu^{2+} at a concentration higher than 200 μM to the solution of 10 μM PrP23–231 caused aggregation of PrP23–231 and influenced the sensorgrams. However, at a concentration of 100 μM or lower, Cu^{2+} neither aggregated PrP23–231 nor decreased the intensities of sensorgrams (Fig. 2E). These results indicate that Cu^{2+} at a concentration range generating no aggregation of PrP23–231 does not influence the interaction between PrP23–231 and the disaccharide unit.

3.4. Anti-prion activity of compounds containing GlcNS6S-IdoA2S

Finally, we examined whether GlcNS6S-IdoA2S is the minimal chemical structure necessary for anti-prion activity. Synthetic compounds composed of this disaccharide unit alone or its multimers exhibited no anti-prion activity at concentrations of 25 μg/mL or higher (Fig. 3). In addition, these disaccharide unit-related compounds did not competitively prevent heparin from inhibiting the formation of abnormal PrP (data not shown).

4. Discussion

Heparin reportedly inhibits the formation of abnormal PrP in prion-infected cells. However, the structural complexity of heparin has hindered detailed structural evaluation of its anti-prion activity. Here, a different depolymerization strategy for heparin made it feasible to explore anti-prion activity in relation to chemical structure. We have shown that LMWHs prepared by

depolymerization with heparinase I have much lower anti-prion activity than those of the same molecular weight prepared by depolymerization with $NaIO_4$, and that heparinase I treatment markedly reduces the anti-prion activity of heparin and LMWHs. These results suggest that the heparinase I-specific disaccharide unit, GlcNS6S-IdoA2S, is an important structure for the anti-prion activity of heparin.

Compared with heparin, GlcNS6S-IdoA2S is remarkably simple and small. Therefore, quantitative analysis of PrP interaction with this structure was successfully conducted using a sugar chip and SPR. The kinetic validity of the interaction was verified by the K_D value (9.7 ± 3.0 μM), which fell well within the K_D value range for general sugar–protein interactions (10^3 to 10^{-1} μM). In addition, the specificity of the interaction of this disaccharide with PrP was demonstrated by competitive inhibition using heparin or pentosan polysulfate. The N-terminal PrP23–89 was responsible for interaction between PrP and the disaccharide unit. The K_D value of PrP23–89 was consistent with that for full-length PrP23–231 and no significant binding signal was observed with PrP89–230. In fact, PrP23–89 includes two distinct regions: the N-terminal positive charge cluster and an octapeptide repeat. Interaction of PrP with the disaccharide unit was dependent on NaCl concentration. This suggests that complementary charge interactions, presumably between negative charges of the disaccharide unit sulfated groups and positive charges of the PrP N-terminal positive charge cluster, contribute to the binding.

In contrast, the octapeptide repeat of PrP might not be involved in binding to the disaccharide unit because, here, the binding profiles of PrP23–231 to the disaccharide unit were unaffected by the presence of Cu^{2+} at a concentration range in which Cu^{2+} did not aggregate PrP23–231. The octapeptide repeat exhibits binding ability to metal ions, particularly Cu^{2+}, which affects the configuration of the flexible N-terminal region [36]. Previous studies have demonstrated PrP interaction with heparin in a Cu^{2+}-enhanced fashion [16,37] or a Cu^{2+}-weakened fashion [17,38]. However, Pan et al. [16] have found that an octapeptide repeat-deleted mutant PrP also binds heparin. They also reported that PrP interacts with heparin through the N-terminal positive charge cluster [16]. Consequently, our data on the PrP binding profiles of the disaccharide unit are consistent with those of heparin determined by Pan et al. [16].

Although the disaccharide unit GlcNS6S-IdoA2S plays an important role in the anti-prion activity of heparin, the disaccharide unit itself and its multimers were not sufficient to exert anti-prion activity. Results of the present study indicate that at least a 4-saccharide unit of $NaIO_4$-depolymerized LMWHs, in which the disaccharide is not abolished, is necessary to exert anti-prion activity comparable with non-depolymerized heparin. Reportedly, O-sulfated glycopyranosides are ineffective compared with polymer-conjugating O-sulfated glycopyranosides for exerting anti-prion activity [39]. Therefore, additional chemical properties near a key saccharide might be required for anti-prion activity.

Regarding structural features involved in the anti-prion potency of heparin, a non-spherical linear conformation as well as negative charge distribution might be necessary for anti-prion activity. Although the GlcNS6S-IdoA2S multimers tested in the present study were highly sulfated and had molecular sizes comparable with LMWHs of 4 or more saccharide units, they did not exert effective anti-prion activity, even at high doses. Because the dendritic distribution of highly charged disaccharide units is likely to cause mutual isotropic repulsion, such GlcNS6S-IdoA2S multimers tested in the present study may have had spherical conformations but not linear conformations. In contrast, LMWHs presumably have a helical rod-shaped conformation [40] because of both the rotational restriction of anomeric linkages and charge repulsion. The linear conformational nature of heparin might facilitate cooperative binding of heparin to abnormal PrP because abnormal PrP also has a rod-shaped fibril structure [41].

In conclusion, our results show that GlcNS6S-IdoA2S, a disaccharide unit of heparin, is a key structure for anti-prion activity, and that it binds to the N-terminal region of PrP. However, additional chemical properties in the vicinity of the disaccharide units of heparin are necessary for anti-prion activity.

Conflict of interest

None.

Acknowledgments

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Transparency document

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